PARTIAL CELL IRRADIATION AND SOFT X-RAY MICRODIFFRACTION

By
V. W. Burns and H. H. Pattee

Progress Report
Prepared under Office of Naval Research Contract
Nonr-225(51) (NR 304-471)

B. L. Report No. 85
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HISTORICAL PREFACE

During the course of this project a number of unpredictable events have taken place which have significantly altered the course of the scientific work. A brief description of these events may help to explain the nature of the present report and assist in evaluation of the somewhat divergent scientific accomplishments of this project.

The original discussions of the Responsible Investigators with representatives of the Office of Naval Research arose, on the one hand, from the long-standing interests of Dr. Burns in cellular dynamics and Dr. Pattee in x-ray microscopic techniques, and on the other, from the desire of Dr. Quimby and Mr. Edelsack of ONR to explore possible biophysical research programs for support by ONR. The first discussions in 1958 took place before the Stanford Biophysics Laboratory was built. Dr. Pattee was then in the Department of Physics and Dr. Burns was in the Department of Radiology at Stanford. An informal proposal for Partial Cell Irradiation with Soft X Rays was submitted to ONR for evaluation in November, 1958.

Following the formation of the Biophysics Laboratory, Dr. Pattee left Stanford for a year of study at the Department of Medical Physics, Karolinska Institute, Stockholm under an NSF Senior Postdoctoral Fellowship. In part through interaction there with Professor Engström, Dr. Carlström, and Dr. Swanbeck, Dr. Pattee became interested in the possibilities of selected-area soft x-ray microdiffraction for studying the molecular organization of intact biological samples. Since the basic soft x-ray techniques and instrumentation required were the same as that proposed for partial cell irradiation, this was considered as a practical extension of the x-ray microbeam technique.

The Contract Nonr 225(51) on Partial Cell Irradiation was started while Dr. Pattee was in Stockholm and was first directed by Dr. Burns and Professor P. Kirkpatrick who originated the x-ray microscope work in the Department of Physics at Stanford.

Following his return to Stanford in February, 1960, Dr. Pattee made the decision to order the Canal Industries electron microsource as the basic equipment for the soft x-ray and cell irradiation work. This

equipment was promised for delivery in 90 days, but was not actually delivered until March, 1962, over two years later. It is fair to say that if it were not for Canal Industries' gross miscalculation on the difficulty in making an electron microbeam system, their instrument would be well worth the cost.

This delay seriously affected the planning of research since Canal Industries' periodic predictions on delivery date were all in error until the last month before delivery. Fortunately, however, there was a considerable amount of scientific work accomplished during this period which was of direct value to the x-ray microdiffraction and partial cell irradiation work. A Cosslett-Nixon type x-ray microsource being used by Dr. L. Zeitz was available for preliminary measurements and design studies, while the investigation of suitable cells and methods for their culture, manipulation, and autoradiographic assay was carried on by Dr. Burns and Mr. Barclay. Permission to extend the scope of the contract to include microdiffraction was obtained from Dr. Shinn of ONR in October, 1960. However, the major published scientific work during this interval was from Dr. G. Swanbeck and Mr. Y. Thathachari, both of whom were supported by this contract. It was their desire to use soft x-ray microdiffraction on their studies of the structure and aggregation of keratin which brought them to this laboratory, but since it never became available, they continued their work with our standard x-ray diffraction equipment.

Dr. Swanbeck's work supported by this contract is described in part in the Annual Summary Report, B.L. Report No. 60, December 1961, and in the paper "The Structure of α-keratin" in Exp. Cell Res. 23, 420 (1961), copies of which have been distributed to ONR.

It was also most unfortunate that the work of Thathachari was cut short by a serious illness which forced his sudden return to his home in India. Much of his diffraction work supported by this contract is described in the paper "Development of Feather Keratin During Embryogenesis of the Chick," J. Cell Biol. 16, 215 (1963), reprints of which accompany this report.

Although the work reported in these papers does not involve microdiffraction techniques, both Dr. Swanbeck and Mr. Thathachari also contributed greatly to the microdiffraction research by their discussions

and calculations. Dr. Swanbeck, with the help of Mr. Belew, constructed a computer program for the Fourier analysis of multiply coiled coils which is valuable for the interpretation of many fiber patterns, and both Swanbeck and Thathachari gave a series of seminars on x-ray diffraction methods.

Since the x-ray microsource is now finally in operation, the work of Dr. Burns on partial cell irradiation and Dr. Pattee on x-ray microdiffraction will take on entirely different approaches to the biological problems involved. The body of this report is consequently divided into a separate section for each subject. In the future, separate reports will be issued by Dr. Burns and Dr. Pattee.

Finally, the contract should be given credit for the indirect support it has provided during the first years of the Biophysics Laboratory at Stanford. Through the basic equipment purchased on this contract and the support as research assistants of graduate biophysics students the scientific contributions of this Laboratory have been greatly aided.

Papers and Publication from this Contract

(a) Swanbeck, G., "The Structure of α-Keratin," Exp. Cell Res. 23, 420 (1961)

(b) Bell, E., and Thathachari, Y. T., "Development of Feather Keratin during Embryogenesis of the Chick", J. Cell Biol. 16, 215 (1963)

(c) Pattee, H. H., "An Apparatus for Soft X-ray, Selected Area, Microdiffraction", Symposium on X-ray Optics and X-ray Microanalysis, Stanford, 1962, Paper 31. (Manuscript in preparation)

X-RAY MICRODIFFRACTION

Background of Interest in Soft X-ray Microdiffraction

The use of x-ray diffraction for the analysis of molecular structure and aggregation in biological material has been a difficult but productive technique for many years. The early work of Astbury, Crowfoot, Bernal, Fankuchen, and others in the 1930's showed the potential of the method on both fibrous and crystalline macromolecules, but it has been only in the last few years that work of Wilkins, Perutz, Kendrew, and others has clearly shown what an enormous amount of information can actually be extracted from large molecules by x-ray diffraction. All this work is carefully reviewed in many publications (see references).

The question naturally arises as to the future developments of x-ray diffraction. What are the essential limits of the method? What additional techniques are most needed for molecular biology? The limits may be in the quality of the diffraction pattern obtainable from a given specimen, or in the effort necessary to interpret the pattern. The additional techniques needed may be in the x-ray instrumentation, or in the preparation of the specimen.

It is an accepted fact that failure to obtain good patterns from what appear to be good crystals or fibers is very likely the result of improper specimen preparation rather than poor x-ray technique. Many good rules have been discovered for handling macromolecular specimens in order to obtain detailed patterns. For example, with crystalline proteins, single crystals must be used no matter how small they may be, and in general wet crystals or fibers give much greater detail than dried material. Many methods of artificial orientation of fibers or anisotropic molecules have been used with more or less success. Nevertheless, some macromolecular species, such as keratin, have never been ordered sufficiently well to yield unequivocal structures. In any case, no matter how well oriented a specimen can be made by artificial preparative methods, there remains the fundamental question, "Is there a difference between the artificially-oriented structure and the same macromolecules under physiological conditions?" Any approaches to answering this question would undoubtedly significantly increase the

value of x-ray diffraction for the central problems of molecular biology.

With regard to x-ray technique there is one well-known limit which hampers most diffraction work with macromolecules--lack of intensity. This results partly from the relatively low electron density of biological material; but it is also the result of looking for the larger spacings of macromolecules, since this requires lower angles and therefore finer collimation. There is also the specimen size limit in many biological fibers or crystals which means a small scattering volume and less scattered energy.

We indicate in the next section how the development of techniques for soft x-ray microdiffraction may be expected to alleviate the intensity and collimation problems, and also how it may help solve problems of the molecular structure or organization of intact biological specimens.

Discussion of Soft X-ray Microdiffraction Methods

Standard x-ray diffraction work is often done with Cu K radiation (λ = 1.54 A) using a sealed-off tube with effective source size of the order of 1 mm^2 and a specific loading of about 1 kw/mm^2. Specimens may be of the order of milligrams for optimum intensity. Although such tubes may be used with microcameras, the collimating conditions are not optimum (Hirsch, 1955).

Commercial x-ray microdiffraction instruments are now available with source diameters from about 40 microns (Hilger Microfocus X-ray Generator; see Stansfield, 1960) down to 10 microns (Rigaku-Denki Co., Ltd. "Microflex"). Nixon (1957) and Carlström (1960) have used the Cosslett-Nixon microsource for microdiffraction of micron-size crystals using specially built cameras. The specimen to film distance in these cameras was several millimeters.

Several reviews of x-ray microdiffraction methods have appeared. Hirsch (1955) has discussed the methods applicable to standard x-ray sources, Cosslett and Nixon (1960) give a concise review of all techniques, and Carlström (1960) discusses the biological applications of microdiffraction. Cosslett and Nixon conclude that microdiffraction results for 50 micron sample sizes are sufficiently encouraging to warrant development of the method down to 5 micron dimensions.

The essential fact which can be derived by a simple analysis of the x-ray image intensity (Huxley, 1953; Hirsch, 1955) is that for the optimum x-ray source and camera design the maximum intensity is independent of the sample size. This may appear surprising since the total energy scattered from the specimen is proportional to its volume; however, owing to the decreased source to film spacing necessary for a given resolution and to the increased brilliance possible with micro-sources, the diffracted image intensity may be kept constant. To make use of this intensity for small specimens requires a film or detector which is capable of recording the small diffraction maxima without undue loss of sensitivity, but this is no problem for specimen areas greater than a few square microns. Nixon (1957) has demonstrated that an x-ray microsource beam of 10 microns diameter can reduce the exposure time by a factor of 50 over standard x-ray tubes. Further speed improvement is possible using optimum camera dimensions as derived, for example, by Huxley (1953). Small-angle microdiffraction is also possible at these dimensions using reflection focusing (Kirkpatrick and Pattee, 1953; Franks, 1958) with great gain in speed. The use of soft x rays allows relaxation of the degree of collimation for a given spacing resolution, and the gain in geometric aperture more than compensates for the lower output of soft x-ray sources. Pattee (1963) has shown that Al K radiation (λ = 8.3 A) can successfully compete in speed with Cu K radiation in a microcamera while permitting resolution of longer spacings. Consequently there is good reason to believe that the intensity problem does not limit the size of microsample which may be used for diffraction, and that in many cases the use of optimum microcamera design may alleviate the intensity problem to some degree.

Furthermore, there is no inherent reason why the sample size could not be reduced to permit the use of naturally-oriented biological samples in the micron region such as single muscle or nerve fibers, single chromosomes, chloroplasts, or sperm heads, or selected areas of thin histological sections where natural orientation may occur. The possibility of obtaining diffraction patterns <u>in situ</u> from selected areas of biological specimens would in many cases help answer the question of possible structural differences between artifically-oriented samples and their natural state. Furthermore, there is good reason to believe

that natural orientation may be better in some microscopic specimens than extracted material which has been artifically reoriented. Finally, microdiffraction methods are essential for studying specimens of inherently small size such as some protein crystals and natural fibers which lose their good orientation in macroscopic samples.

The Design of X-ray Microdiffraction Source and Camera

If we are not limited to fixed focus x-ray tubes or to any fixed dimensional restrictions we may easily derive, following Huxley (1953), what the optimum source and camera dimensions are, given the desired maximum spacing to be resolved and the size of the specimen. Following the notation shown in Fig. 1 we may write the flux per unit area of image at the film as

$$I_f = I_0(K_1 a^3) \frac{f^2}{a^2} \frac{1}{(s+d)^2} \qquad (1)$$

where I_0 is the specific intensity of the x-ray source
K_1 is the volume scattering coefficient of the specimen
f is the diameter of the x-ray source
a is the collimating aperture diameter
s is the source to specimen distance
d is the specimen to film distance.

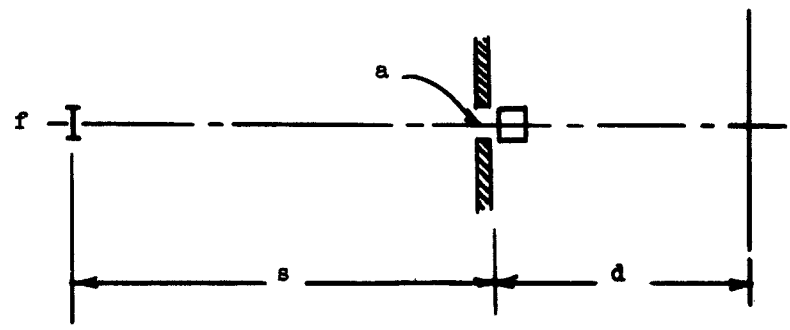

Figure 1

For a source diameter larger than $f = d(s + d)/a$ there is no increase in the spot intensity so we may call this the collimation requirement. If the camera is to separate adjacent points in the reciprocal lattice separated by the spot size F, where

$$F = \frac{a(s + d)}{s} + \frac{fd}{s}, \qquad (2)$$

then $d/Y \leq F$, where Y is the maximum resolvable spacing in units of the x-ray wavelength. Substituting this resolution requirement into the collimation requirement gives $f = s/2Y$ for maximum intensity independent of sample size.

We now use the fact that the maximum specific intensity of a microfocus tube is inversely proportional to the source diameter so that $I_0 = K_2/f$. By substitution of these restrictions in Eq. (2) we obtain an expression I_f as a function of f and a only which can then be differentiated and solved for the extrema. This gives the solution $f = 3a$ for maximum intensity. We then have the conditions for maximum intensity for a given specimen size and desired resolution as $f = 3a$, $s = 2fY$, and $d = 2s$. Furthermore, the maximum intensity under these conditions is independent of sample size. The maximum intensity does depend on the inverse square of the resolution Y, however.

Use of Soft X rays

The analysis of the optimum wavelength for a given sample size and resolution is not worthwhile in general since so few good target materials exist for the soft x-ray region. Titanium and aluminum have proven to be excellent for intense microsource targets. Titanium has a K wavelength of 2.76 A, or 1.8 times the wavelength of copper K, and a higher melting point, but its heat conductivity is relatively low so that the maximum loading is slightly less than copper. Aluminum has a K wavelength of 8.3 A, or about 5 times the wavelength of copper, with a lower melting point and heat conductivity, so that its maximum specific loading is about one-third that of copper.

One advantage of softer x rays which is difficult to measure quantitatively is the greater ease with which good collimating apertures may be made. This results from the stronger absorption of the soft x rays

which allows the use of much thinner collimator disks and a consequent reduction in the difficulty of producing well-shaped small collimating holes. For copper radiation the width of the collimator must be about 10 times that for aluminum for the same attenuation of the primary beam.

The advantage of using soft x rays to increase speed may be illustrated by an example using the optimum intensity conditions given at the end of the last section. If we wish to resolve a 60 A spacing in a specimen with a side of 2 microns using a wavelength of 1.5 A, we find that the source should be 6 microns diameter, s = 480 microns, d = 240 microns, so that the total camera length is 720 microns, or about 3/4 mm . If we wish to increase the resolution to 600 A under the same conditions the camera length would have to be increased to 7.2 mm and the exposure time would be 100 times longer. However, by using a wavelength of 8.3 A (Al K) the 600 A resolution could be obtained by doubling the camera length with a corresponding loss of geometric speed of only a factor of four. The specific intensity loss from the Cu to Al target will amount to a factor of 3 to 5, so that in any case a considerable gain in speed is possible by using soft x rays.

This neglects the effect of specimen absorption which will be greater for the soft x rays. In macroscopic specimens the loss of intensity using Al K radiation would be prohibitive, however, in the above example the specimen thickness is well under the half-value thickness for aluminum radiation so that no absorption loss of diffracted intensity occurs from the use of soft x rays in this case.

Construction and Operation of the Microsource and Microcamera

In order to achieve optimum intensity for a variety of microdiffraction specimens it is necessary to have a source and camera with wide ranges of adjustable parameters as indicated by the design conditions. At the same time, the problems of alignment, specimen and beam control, and stability must be simple enough to control to allow routine microdiffraction work.

The basic electron microprobe used in this apparatus is manufactured by Canal Industries, Inc., Bethesda, Maryland. It provides all the necessary vacuum and electronic controls and the two-lens electron

optical system. The insulation is designed for 80 kv with all power supplies regulated to provide long-term stability for micron-size beams. In order to provide the maximum beam intensity and yet provide adequate working space in the vicinity of the focal spot, the upper pole piece was removed. The electron lens then consists of the lower pole piece (19) and the upper shroud (6). Under these conditions it is possible to operate under strong excitation with the consequent low spherical aberration, and still have a 1.5-inch diameter working area at the focus. Of course no magnetic material can be used in this area since it is in the objective magnetic field. The working area is shown schematically in Fig. 3. The electron beam (16) from below is imaged on the thin foil target (22) which is fastened with conductive glue to the target disk (23). This target disk is held in position by the vacuum. Targets may be changed in 5 minutes. Directly above the target foil (22) is a ribbon (24) held at both ends by a horseshoe-shaped mount (21) rigidly fastened to the aperture micromanipulator. This ribbon is held in contact with the target foil, and its thickness determines the source-to-aperture distance, s , since the aperture is mounted directly on top of this ribbon. The specimen may be mounted a variety of ways. If no humidity control is necessary the specimen may be placed on the bottom of a plastic cover slip over a small hole. The specimen micromanipulator (31) can then be used to position the region of the specimen to be irradiated directly over the beam. The film (27) may be placed directly on top of the same cover slip which holds the specimen, the thickness of the cover slip determining the specimen-to-film spacing, d . For specimens which require humidity control, a microchamber has been constructed (with Dr. D. Kaiser) which has two thin plastic windows to allow passage of the soft x rays, and input and output tubulation for continuous gas flow. For larger spacings the target-film (30) and target-specimen (32) adjustments are used.

All of the alignment operations are done under the incident-light microscope shown in Fig. 2 which is rigidly mounted coaxially with the electron beam. The microscope has a calibrated micrometer eyepiece (14) and a calibrated fine-focus adjustment (13) so that all positions and sizes in the region from the electron aperture (20) to the film are directly measurable in microns. The x-ray beam is viewed by inserting

a fine-grained fluorescent screen or a fluorescent crystal at the focus of the microscope. The beam focusing procedure depends on the problem at hand and the recent history of operation. During exposure, the target current is continuously monitored. The target disk is electrically isolated from the rest of the instrument so that only the electrons passing through the electron objective aperture will be measured. Beam drift or instability may therefore be checked and corrected during exposure. All alignment and exposure of film is done in red light, so that no light-tight enclosures or cassettes are needed. Since the film must necessarily be very fine-grained, there is no problem of light fogging the film during alignment.

The choice of film is determined by the required resolving distance d/Y, since this must be larger than the resolution of the film. At present we have found Eastman AR-10 stripping film to be adequate for most soft x-ray work. It has an emulsion thickness of only 5 microns which provides some filtering by passing the harder components. It will also resolve 2 microns, and has very good speed relative to other fine-grain films. Liquid emulsion may also be used for thinner emulsions and better filtration for soft x rays.

Figure 4 shows typical x-ray microcamera dimensions roughly to scale for 10 micron sample size and a specimen spacing resolution of 10 wavelengths. The optimum focal spot diameter, f, should be somewhat less than $3a$ since the target-to-specimen distance s' is longer than the target-to-aperture distance s. The derivation of optimum dimensions did not take the aperture thickness into account. A gold aperture of this thickness would be more than adequate for titanium K radiation.

FIGURE 2 - LEGEND

1. Filament
2. Grid
3. Anode
4. Condenser lens shroud
5. Detail of this area shown in Fig. 3
6. Objective lens shroud
7. Electron objective lens aperture positioner
8. X-ray microcamera insert
9. Film position micromanipulator x-axis control
10. Film position micromanipulator y-axis control
11. Light microscope objective
12. Incident light illumination source
13. Calibrated vertical fine-focus adjustment
14. Eyepiece crosshair micrometer
15. Viewing eyepiece

FIGURES 3 and 4 - LEGEND

16. Electron beam
17. Electron aperture control rod
18. Electron objective lens inner shroud
19. Electron objective lens lower pole piece (no upper pole piece)
20. Electron objective aperture
21. X-ray aperture mounting frame
22. X-ray target foil
23. X-ray target disk
24. X-ray aperture ribbon
25. X-ray aperture disk
26. Specimen holder
27. Film holder
28. Detail of this area shown in Fig. 4
29. To film position micromanipulator
30. Target-film spacing adjustment
31. To specimen position micromanipulator
32. Target-specimen spacing adjustment

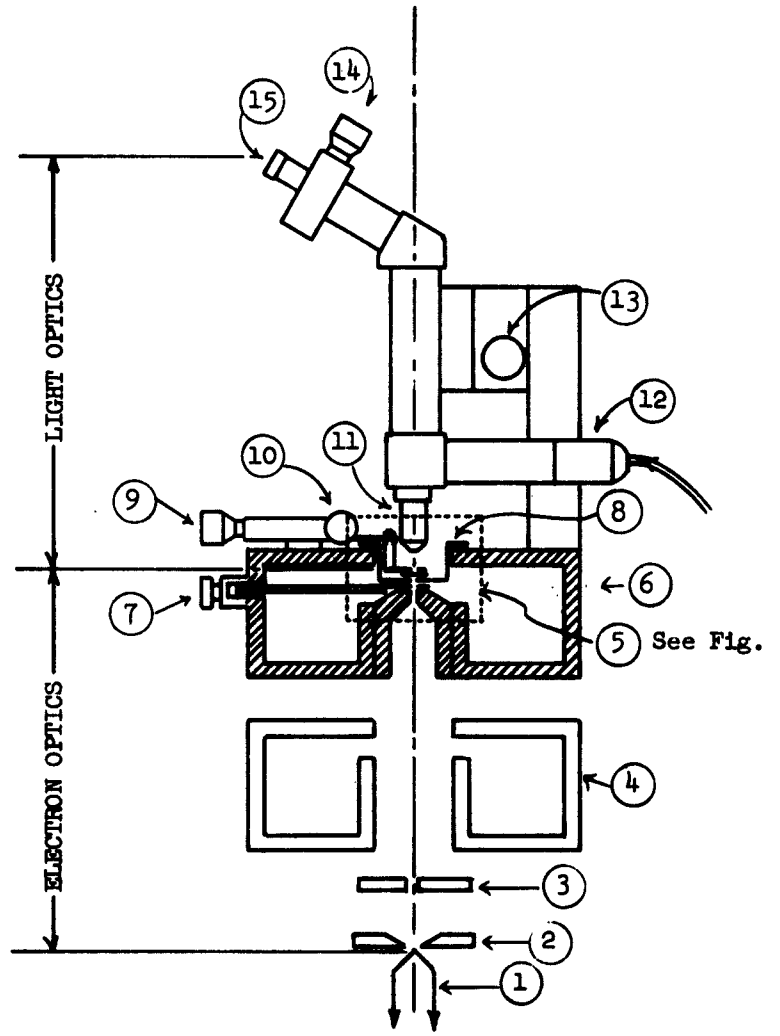

Figure 2

X-RAY MICROBEAM-ELECTRON AND

LIGHT OPTICAL SCHEMATIC

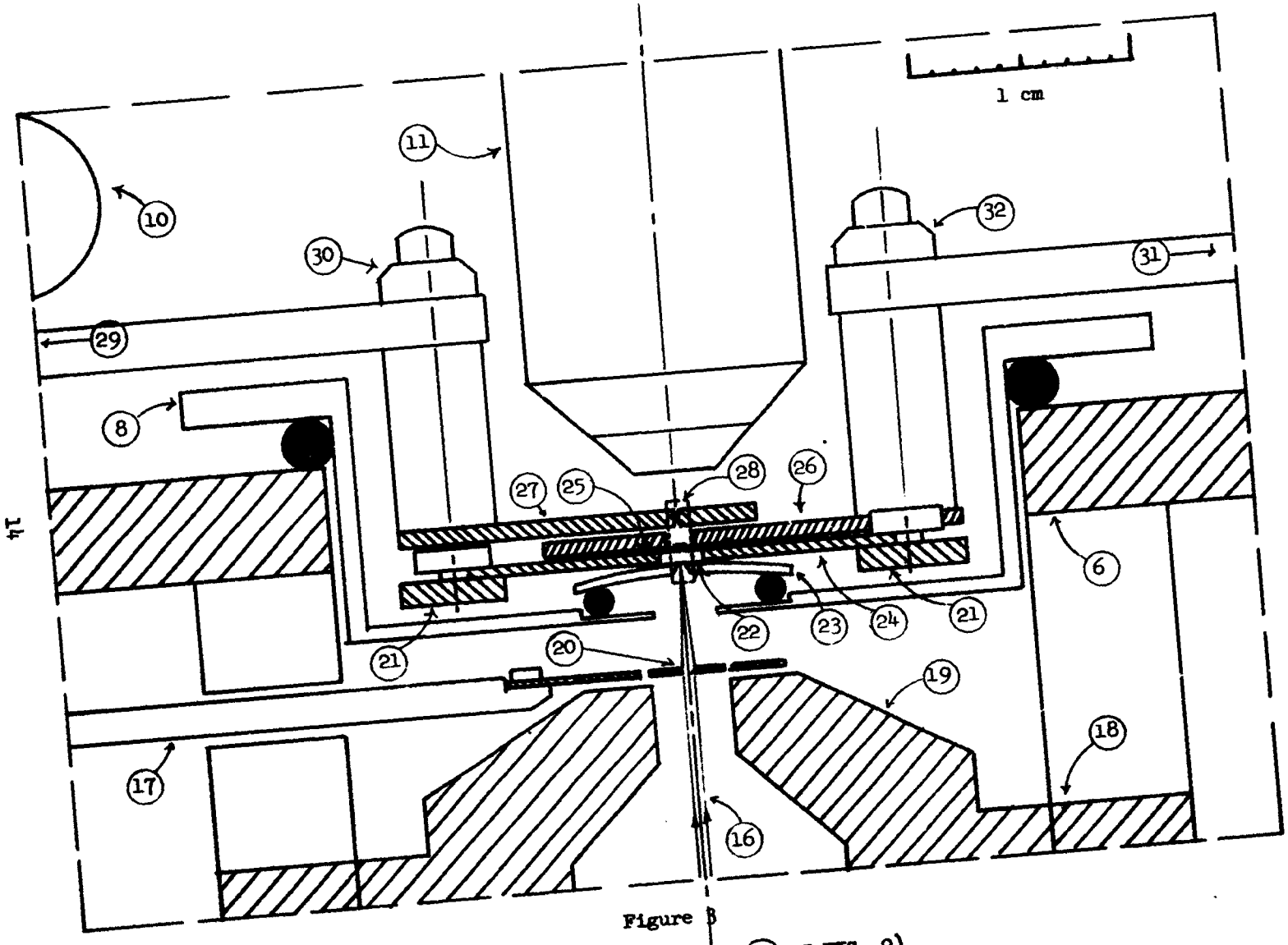

Figure 3

X-RAY MICROCAMERA (AREA (5) OF FIG. 2)

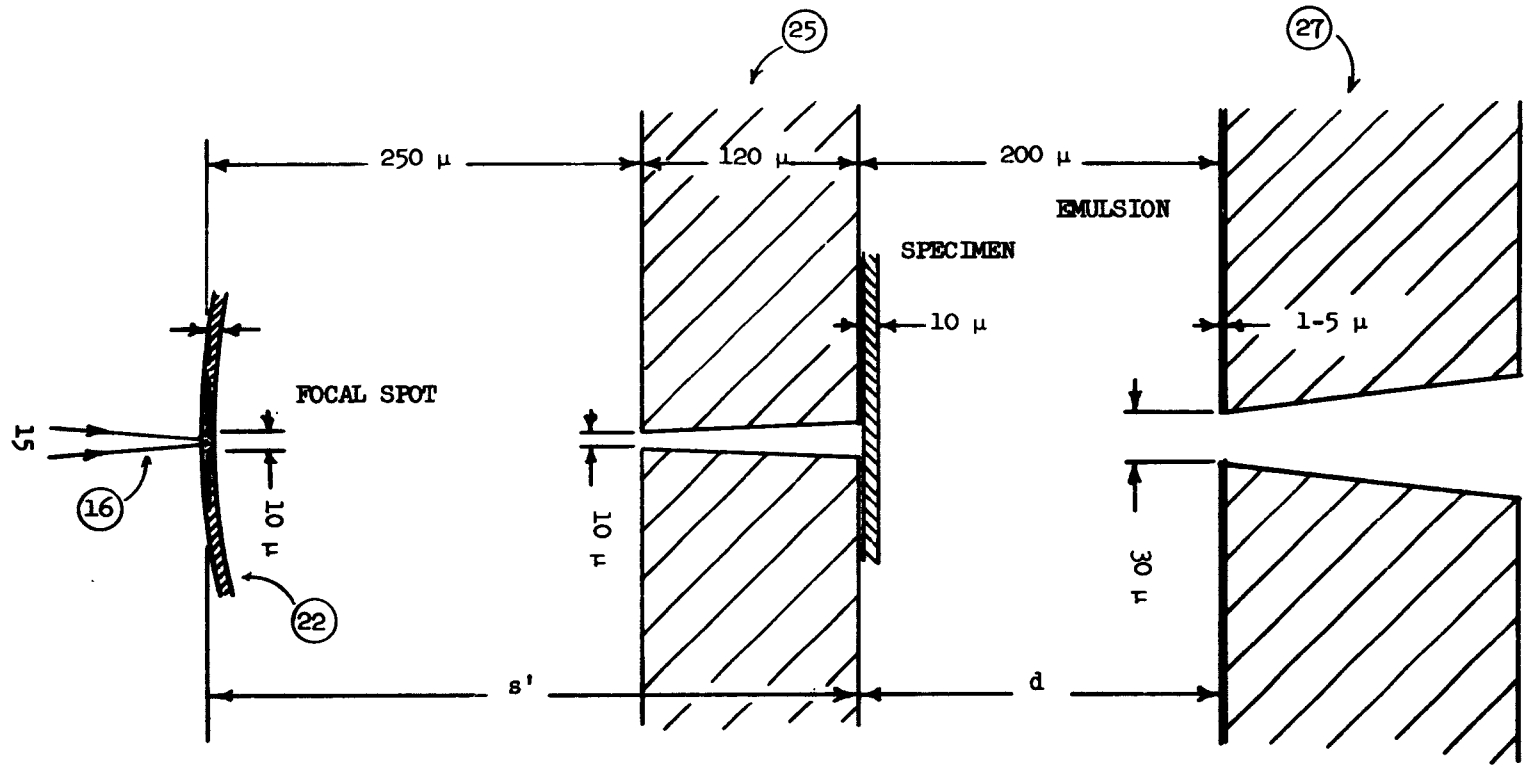

Figure 4

X-RAY MICROCAMERA TYPICAL DIMENSIONS

(Area (28) of Figure 3)

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BIOLOGICAL MATERIALS AND METHODS

FOR PARTIAL CELL IRRADIATION APPLICATION

Introduction

The fresh water alga Micrasterias radiata was chosen for investigation of its suitability for microbeam work. This alga is a free-living form which requires only inorganic salts for nutrition. It is easy to handle and its nutrient environment is of completely determined composition; also single cells divide readily and indefinitely, there is no need to put up with the complications of cellular interactions as encountered in systems where many cells must be together in order to get division of any of them. Many strains and species of Micrasterias exist, some with well-defined chromosomes and otherswith well-defined nucleoli. M. radiata belongs to the latter class and is particularly notable because it has a single large uniform-sized spherical nucleolus. It is expected that this tidy structure will lend itself well to complete destruction by a circular microbeam of constant diameter such that a minimum of extra-nucleolar material experiences radiation damage. The interpretation of the work of other workers who have attempted selective destruction of the nucleoli of cells is severely restricted because their cells have contained many nucleoli only a fraction of which were usually irradiated, and the size and shape of each nucleolus has varied greatly so that in some cells the selected necleolus may not be uniformly irradiated and in others an excessive amount of extra-nucleolar material may be irradiated. With such biological material the destruction of nucleolar function by a fixed microbeam irradiation treatment varies enough from cell to cell to obscure the nature of nucleolar function when analyses of statistical significance are made. M. Radiata would seem to circumvent these difficulties and it was therefore chosen despite the fact that its behavior and physiology are not well known. In order to use this cell more extensive investigation of behavior and physiology has proven necessary than would have been required if a more familiar species had been chosen.

Areas of Investigation

M. Radiata was investigated first in order to answer the following questions to lay a foundation for its use in microbeam work:

(a) What environmental conditions give uniform, rapid reproducible growth? Variables of interest here are temperature. intensity of illumination, durations of light and dark periods, composition of medium, effect of carbon dioxide.

(b) What are the morphological details and the time sequence of events of the division cycle?

(c) Can the environment be manipulated to give synchronous division of a population without producing abnormal division? Since the nucleolus is not visible throughout the division cycle and its radiation sensitivity and its function may vary with stage of division, it is desirable to select cells for irradiation in a known stage of division. If many cells are to be irradiated in a single experiment in order to produce statistically significant data, the time required for selection of cells in a single stage of division from a randomly dividing population becomes prohibitive. If however most members of the population are dividing in synchrony, it becomes an easy matter to select 30 to 50 individuals in the same stage of division.

(d) What methods of assaying the effects of microbeam irradiation of the nucleolus are suitable?

(e) What is the sensitivity of the alga to whole cell irradiation? The answer to this question will help to decide what dose rates are likely to be asked of the microbeam apparatus.

Results of Experiments to Date

(a) Keeping temperature constant at 23° C and other conditions constant, the growth rate for various light intensities was determined. This required construction of a special incubator large enough to house the four fluorescent light tubes used for illumination but with good temperature control near to the ambient temperature range. The cells were grown in depression slides covered with neutral density filters. Illumination levels varying from 100 to 600 foot-candles were employed. For levels greater than 300 foot-candles the number of individuals per

culture increased linearly with time. At lower levels the number increased at the same rate at first and then at the end of a week's incubation, it began to level off. Morphologically abnormal forms appeared after growth under low intensity illumination. Generation time under the higher intensities was on the order of 3 days. It seems unlikely that we will be able to reduce this time appreciably. There are advantages in working with such long generation times however, one of them being that the division stage of cells selected for irradiation changes relatively slowly with time so that slow operations, such as selection by micromanipulation, can be employed profitably.

These experiments were carried on using an illumination cycle of 12 hours of light and 12 hours of darkness. Other workers have shown that continuous illumination gives lower growth rates and eventual degeneration in the genus Micrasterias. We have found that at intensities of 500 to 600 foot-candles the majority of cells divide during the last third of the light cycle. Those cells that are going to divide on a given day, amount to about a third of the total number.

(b) The nucleus tends to be obscured by the chloroplast except during cytokinesis. For this reason we have no good observations of karyokinesis, since cytokinesis and karyokinesis are not simultaneous. The nuclei and nucleoli are very clear under phase contrast during cytokinesis. Before cytokinesis a nucleolus covered by the chloroplast can often be discerned in each semi-cell, about the radius of the nucleus away from the central fissure. The less easily discernible nuclear membrane may be present at this time but cannot be demonstrated. Some observations suggest that later on granules gather near the nucleolus and a nuclear membrane is then formed. As the semi-cells separate and the cytoplasmic bridge (isthmus) elongates, the nucleoli migrate into the newly forming semi-cells. About one hour after cytokinesis begins the nucleoli are clearly visible with nuclear membranes surrounding them in the new semi-cells. This would be a stage most favorable for micro-beam irradiation of the nucleoli. From two hours onward the chloroplast invades the new semi-cells and gradually obscures the nuclei and nucleoli. The new semi-cells grow to nearly full size within 8 hours after the start of cytokinesis. These events of the division cycle are shown in the accompanying time sequence of 12 photographs. An additional photograph

PATTERN OF CELL DIVISION IN MICRASTERIAS RADIATA

All Photographs made with phase contrast. Magnification of Figs. 1 through 12 is 600 X.

Fig. 1 Early stage of division in which first dim outline of the nucleus of each semi-cell beings to appear. (5 min.)

Fig. 2 The isthmus has elongated, the semi-cells have separated slightly, and the division furrow in the isthmus has become visible. (12 min.)

Fig. 3 Faint indication of the nucleolus appears in one semi-cell near the isthmus boundary. (15 min.)

Fig. 4 Continued enlargement of the isthmus. (32 min.)

Fig. 5 Nucleolus in left semi-cell clearly visible. (55 min.)

Fig. 6 Lobes of isthmus begin to expand. (65 min.)

Fig. 7 Nucleolus is clearly visible as dark circle within lighter elliptical nucleus of each semi-cell. (73 min.)

Fig. 8 Chloroplasts begin to infiltrate the new semi-cells. Entire boundary of nucleus becomes visible. (90 min.)

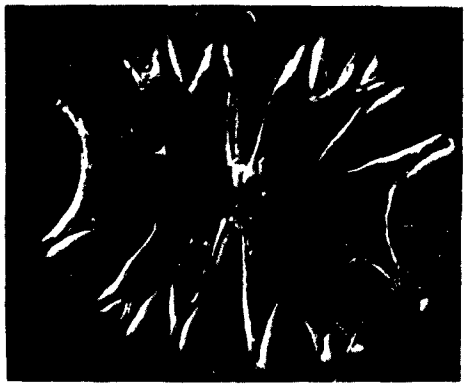
Fig. 9 Nucleoli show with maximum clarity. (105 min.)

Fig. 10 Chloroplasts begin to obscure nuclei and nucleoli. (120 min.)

Fig. 11 Cells separate. Lobes begin to overlap. (135 min.)

Fig. 12 Chloroplasts completely obscure nuclei and nucleoli. (150 min.)

Fig. 13 Enlargement (1000 X) of isthmus during period of maximum clarity of nuclei and nucleoli.



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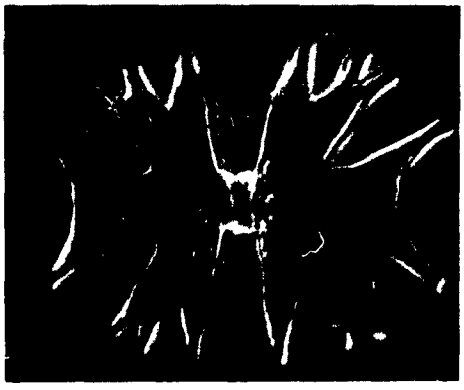

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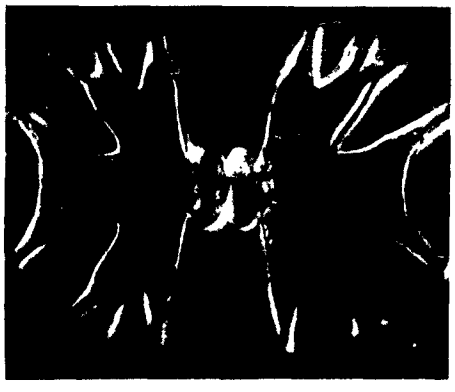

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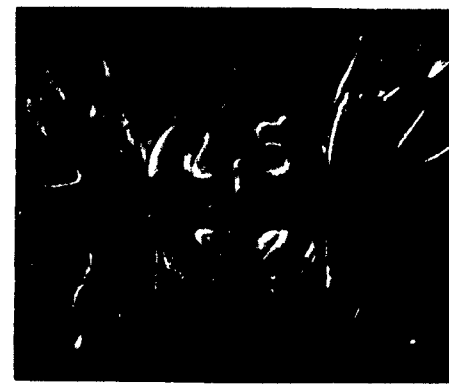

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at higher magnification shows very clearly the nuclei and nucleoli at about 90 minutes after beginning of cytokinesis.

(c). Synchronous division was attained by letting the cells grow to high concentration, then subjecting them to low temperatures and low light intensities followed by addition of new media and return to the original conditions of illumination and temperature. The experiment was as follows: On day zero a single cell was started in a depression slide at 23° C, 420 foot-candles, and 12 hours light, 12 hours dark illumination cycle. Forty-three days later the culture was put at about 17° C, 270 foot-candles. There were about 500 cells in the culture at this time, descended from the single cell inoculum. The division rate dropped off under the new conditions, until at 55 days it had dropped to zero. The old medium was then removed and new added. The culture was returned to 23° C and 420 foot-candles. At 57 days more than 90% of the cells divided during the final four hours of the light period. Since these divisions would normally be spread over about 72 hours or about 5% dividing during any 4 hour interval, the degree of synchrony induced by the treatment is quite significant. After day 57 the degree of synchrony declined rapidly.

Subsequent attempts to reproduce the synchronization of the above experiment have been only partially successful. Recent work of Hase (1962) and Tamiya (1963) on the synchronization of the alga Chlorella points the way to the understanding and control of synchronization in Micrasterias. These workers have found that sulfate is specifically required for algal cell division probably because certain sulfur containing compounds--sulfur containing peptide nucleotides, sulfur containing deoxypentase polynucleotides, and coenzyme A--must be elaborated before division can take place. Cells which ripen in a sulfate-free medium can be induced to divide by the restoration of sulfate. The relevance of this mode of division control to Micrasterias is under investigation. In addition Tamiya found that the use of an illumination cycle of 17 hours of light followed by 9 hours in the dark at 25° C in the presence of ample sulfate would lock division in phase with the illumination cycle so that all cells would divide during the first hours of darkness in each cycle. Experiments directed to obtain an appropriate illumination cycle for Micrasterias are underway. If these are successful,

a predictable and reliable supply of dividing cells will be available for microbeam irradiation.

(d) Various methods of assaying the effects of nucleolar irradiation have been considered and the conclusion was reached that the use of appropriate tritium labeled tracers followed by quantitative autoradiographic determination of the positions and amounts of tracer in the cell is the single most versatile and specific technique available. The evidence of other workers using different kinds of cell than ours indicates that the nucleolus is involved in ribosomal RNA synthesis (Perry, 1962) and in nucleoprotein synthesis (De, 1961). Tritiated cytidine or uridine can be used to label the nucleic acids specifically, and tritiated basic amino acids to label the proteins of the nucleus, and the nucleohistone fraction can be identified by suitable extraction procedures. The use of pulse labeling can help to discriminate among different RNA fractions which turn over at differing rates.

No studies of the uptake of labeled nucleosides or amino acids or of the autoradiography of Micrasterias are recorded in the literature. Our first study was simply to determine if tritiated uridine is taken up rapidly by Micrasterias. Uptake was measured by assay of the activity of a large number of cells in a liquid scintillation counter. At concentration as low as 10^{-8} M uridine was taken up so rapidly that it appears feasible to label with short pulses of uridine and still be able to detect the labeled molecules autoradiographically.

The next series of studies were directed to the use of autoradiography with Micrasterias. Since cytological techniques (fixation, staining, etc.) have not been described in the Micrasterias literature, these had to be developed. A persistent problem has been the disinclination of these single free-living cells to adhere to glass or other surfaces. Early tests with adhesives failed to uncover an appropriate one. It was found that the cells could be collected on Millipore filters, fixed and stained and transfered from the filter to slides for observation, but this handling tended to lose some cells and distort the structure of others. Nevertheless it was an efficient method for testing various nucleolar stains. Although all the standard formulations for methyl green-pyronin staining were applied, none was found to stain the Micrasterias nucleolus effectively. Next cells collected by

filtration and pressed onto slides were investigated autoradiographically using the stripping film technique. It proved impossible to transfer the film to the slide without losing cells. Some tests of the use of very thin films of collodion to hold the cells down were partially successful but a film thin enough not to absorb an appreciable fraction of tritium beta particles is fragile and difficult to apply. Finally an adhesive of ovalbumin and glycerine was tested and found to hold the cells throughout fixation and autoradiography. Even with the use of this adhesive it is necessary to keep the cells wet at all times during processing to prevent curling up of the fine points of the star-like cell wall. Various fixatives have been tested for ability to preserve cell structures such as the nucleolus intact throughout processing. Ethanol containing 0.3% acetic acid is effective. A better fixative is De's formalin-ethanol-acetic acid (FAA).

Preliminary autoradiographic experiments have been done as follows:

(1) Cells partially synchronized with about 5% dividing at start of experiment.

(2) Apply H^3 uridine of specific activity 2.7 c/m M at a concentration of 1 microcurie per ml. Incubate in light for 30 minutes.

(3) Apply cells to slide previously treated with ovalbumin adhesive.

(4) Bring to near dryness then fix in FAA, wash in 70% ethanol.

(5) Dip in Ilford G-5 liquid emulsion.

(6) Expose for two weeks.

Results: Many cells seem to be labeled throughout the cytoplasm as judged by the dense grain clusters, with higher grain density appearing over the isthmus where the nucleus and nucleolus is located. These experiments demonstrate that Micrasterias incorporates uridine supplied externally efficiently into the nucleic acids, contrary to the experience with some other species of algae. Also these experiments mark the first solutions to the tedious small problems of adhesion, fixation and emulsion application which turned out to be rather difficult for Micrasterias.

(e) In order to estimate the approximate dose which will be needed in microbeam experiments, the radio-sensitivity of Micrasterias to 200 kv x rays was determined. 5000 rontgen produces appreciable division delay and lethality. Current data on the intensity of the microbeam

indicate it is capable of delivering 5000 r in a few seconds. Therefore, assuming that a dose of the same order of magnitude will inactivate the nucleolus, the time required to irradiate each nucleolus will be short and it should be possible to irradiate many cells in the same stage of division during the course of a single experiment.

Miscellaneous Techniques

Selection of cells for microbeam irradiation and for single-cell cultures requires the use of a micromanipulator. An hydraulic bellows type was purchased. To select and pick up cells bent capillary pipettes were made and fitted to the micromanipulator head. A microsyringe connected to the capillary pipette provides controlled suction. Using this system under a stereomicroscope individual cells can be picked up and transferred to depression slides or other holders.

The problem of support of the cells during and after irradiation has been considered. With the apparatus designed for microbeam work it should be possible to irradiate 20 or 30 cells in rapid succession if they are all on one support. It will be necessary to be able to find and identify each cell after irradiation for subsequent observation. Micrasterias does not attach well to glass or plastic and so tends to move under the influence of convection currents, etc., making identification in terms of position coordinates of each cell at the time of irradiation difficult. To solve this a grid cage slide has been developed. This consists of a thin plastic sheet with tiny holes bored in it every 2 mm or so, and attached to a slide. With the micromanipulator cells can be selected in a given stage of division, picked up and deposited one to each hole, the whole then being immersed in nutrient medium. The cells cannot get out of the holes and so individuals can be followed without fear of confusing one with another.

The methods of handling cells outlined in the above two paragraphs are unfortunately slow and laborious. Micromanipulation could be dispensed with entirely if (a) synchronously dividing cells can be made available and (b) they can be made to stick in place on mylar foil without the use of a grid cage. Synchronization has been discussed in an earlier section. As for (b) it has been found that ovalbumin adhesive will stick to mylar and will attach the cells nearly as well as

it does to glass slides. Some trouble may be experienced in making the adhesive layer uniformly thin enough to prevent serious absorption of the microbeam during irradiation. Mylar foil with cells affixed has been successfully removed from a mock-up irradiation holder, affixed to a slide and carried through fixation, washing, and emulsion-coating processes without substantial loss of cells.

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